Construction of a Chickpea Linkage Map and Its Comparison With Maps of Pea and Lentil

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An integrated genetic linkage map of chickpea (*Cicer*) has been developed that consists of 9 morphological, 27 isozyme, 10 RFLP, and 45 RAPD markers covering 550 cM. The map was made from segregation data from populations of three interspecific crosses of cultivated chickpea (*C. arietinum*, $2n = 16$) and a closely related wild species (*C. reticulatum*, $2n = 16$). The linkage map has 10 linkage groups representing the eight chromosomes of chickpea. Interspecific crosses were chosen for mapping because of the extremely low level of polymorphism found within the cultivated chickpea species. Several regions of the genome were found to be slightly skewed from the expected Mendelian ratios of alleles. The map was compared with published maps for pea (*Pisum*) and lentil (*Lens*). Five regions of the chickpea map have gene orders that are similar to those found in the pea genome. The degree of similarity is somewhat less than that found between pea and lentil, which is consistent with the evolutionary distances between these three genera. We have also observed that lentil genomic DNA RFLP probes hybridize poorly to chickpea DNA, indicating considerable divergence of these genomes at the sequence level.

Chickpea (*Cicer arietinum* L.) is a self-pollinated diploid ($2n = 2x = 16$) annual grain legume that is an important food crop in the Indian subcontinent and Middle Eastern regions. Genetic analysis of chickpea has so far included only morphological and, recently, isozyme variation (Gaur and Slinkard 1990a,b; Kazan et al. 1993; Muehlbauer and Singh 1987). Linkage analysis, which is proving to be an important tool for improving the productivity of other crop species, has been limited by the lack of usable polymorphism found within chickpea. Wide variation for morphological traits exists within *C. arietinum*, but few linkages have been reported (Muehlbauer and Singh 1987). In addition, isozyme polymorphism within the species has been found to be infrequent (Oram et al. 1987; Tuwafe et al. 1988). Gaur and Slinkard (1990a,b) and Kazan et al. (1993) recognized the low level of polymorphism in the species and performed isozyme analysis on populations derived from interspecific crosses between *C. arietinum* and *C. reticulatum* L., the presumed progenitor of *C. arietinum* (Ladizinsky and Adler 1976). By this approach, Gaur and Slinkard (1990a,b) were able to develop a linkage map of 13 isozyme loci on four linkage groups.

With the advent of restriction fragment length polymorphisms (RFLPs) (Botstein et al. 1980) and random amplified polymorphic DNAs (RAPDs) (Williams et al. 1990), large numbers of polymorphisms are now available for linkage analysis. These tools are being used to develop detailed linkage maps of many crop species to assist in plant breeding [see Tanksley et al. (1989) for a review]. Preliminary investigations of chickpea using RFLP and RAPD techniques have revealed that the degree of polymorphism within *C. arietinum* is very limited, as was the case for isozyme polymorphism. With this information, we elected to focus our DNA mapping efforts on *C. arietinum* × *C. reticulatum* crosses, as Gaur and Slinkard (1990a,b) and Kazan et al. (1993) did with their chickpea isozyme studies. In an effort to best integrate all of the published efforts with chickpea, we obtained the precise populations used in previous studies from Kazan et al. (1993) and Gaur and Slinkard (1990a,b), and used those populations to expand the chickpea map using DNA polymorphisms.

Material and Methods

Plant Materials

Mapping populations were obtained from Gaur and Slinkard (1990a,b) and Kazan et

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al. (1993). They consisted of three interspecific crosses of *Cicer arietinum* × *C. reticulatum*. Parents of the crosses from Kazan et al. (1993) were *C. arietinum* kabuli type (P.I. 360177) × *C. reticulatum* (P.I. 489777) and *C. arietinum* desi type (P.I. 360348) × *C. reticulatum* (P.I. 489777). The Gaur and Slinkard (1990) cross used was *C. arietinum* (ICC 4957) × *C. reticulatum* (P.I. 489777).

F_2 plants were sampled for DNA extraction from the Kazan et al. populations. For the Gaur and Slinkard (1990a,b) cross, 10 F_3 family seed were sown and individual F_3 plants were sampled for isozyme analysis. Plant tissue from all members of each F_3 family was pooled for DNA extraction.

Morphological and Isozyme Analysis
Morphological and isozyme analysis was repeated on these populations by the methods first performed by the original authors (Gaur and Slinkard 1990a,b; Kazan et al. 1993). The original findings were confirmed, and in the case of the Gaur and Slinkard population, one additional isozyme—G6PD—was found to be segregating and was scored.

DNA Isolation
DNA was isolated by an extensive modification of the method described by Murray and Thompson (1980). Young leaf material was collected from greenhouse-grown plants. Petioles were removed and fresh leaf material was used directly for the extraction. One gram of each leaf sample (fresh weight) was submerged in liquid for 1 min and then ground to a fine powder. The powder was quickly transferred into a 50 ml centrifuge tube containing 7.5 ml of ice cold extraction buffer (0.35 M sorbitol, 0.1 M Tris, 5 mM EDTA, pH 7.5). The tube was capped and briefly shaken, 7.5 ml of nuclei lysis buffer (2 M NaCl, 0.2 M Tris, 50 mM EDTA, 2% CTAB, pH 7.5) was then quickly added, followed by 3 ml of 5% sarkosyl solution. The tube was capped and shaken vigorously. Ground samples were kept on crushed ice until all samples of the set were ground.

Sample sets were incubated in a 65°C water bath for 20–60 min. After incubation the tubes were allowed to cool for a few minutes at room temperature and 18 ml of chloroform/isoamyl alcohol (24:1) was added to each tube. After being capped and briskly shaken, the tubes were centrifuged at 500× G for 15 min. The resulting aqueous layer (top) was transferred to a fresh tube, extracted again with 15 ml chloroform mixture, and centrifuged for another 10 min to separate phases. The aqueous layer was removed, taking care to avoid debris at the interface, and placed into a fresh 50 ml tube. Two volumes of −20°C, 95% ethanol were added to the aqueous phase, and the tube was capped and slowly inverted until well mixed.

Precipitated DNA was collected by centrifugation at 500× G for 10 min. Supernatant was poured off, and the DNA pellet was rinsed in 70% ethanol and dried before it was suspended in 1 ml of TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA).

RFLP Analysis
Eight to ten microgram aliquots of parental DNA were restricted separately for 14 h with six restriction enzymes (*Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Xba*I) according to instructions from the manufacturer (BRL, Bethesda, Maryland) and separated on 0.7% agarose gels at 0.5 v/cm. DNA in the gels was visualized by ethidium bromide staining and was transferred from the gel onto nylon membranes (NEN GeneScreen Plus) in alkaline solution as described by Reed and Mann (1985). DNA from all members of the progeny populations was also restricted with each of the enzymes and blotted onto membranes. Membranes were probed with cloned and oligolabeled (Feinberg and Vogelstein 1983) lentil random cDNA and genomic DNA inserts [obtained from Havey and Muehlbauer (1989)] using methods of Maniatis et al. (1982). Filters were washed under low stringency conditions (2× SSC, 0.1% SDS, 65°C) and placed on Kodak XAR-5 X-ray film with DuPont Cronex Lightning Plus intensifying screens at −70°C for 2–3 days. Probes showing polymorphism between the parents for the position of the hybridization signal for one or more enzyme digests were subsequently used to evaluate the progeny populations. Progeny were scored as having the signal mobility of either or both parents. RFLP probes reported in this article are available from the authors, and information regarding the enzymes used to discover polymorphisms can be supplied with the probes upon request.

RAPD Analysis
RAPD analysis was performed with 70 10-mer primers by a modification of Williams et al. (1990). Each 25 ml reaction contained 1 unit of Promega *Taq* polymerase, Promega reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, and 0.1% Triton X-100), 0.1 mM of each dNTP, 2 μM primer, and approximately 35–50 ng template DNA. Thermal cycling took place in a Perkin Elmer Cetus Gene Amp PCR system model 9600. Forty cycles of amplification were performed, each consisting of 20 s of denaturing at 94°C, 1 min of annealing at 36°C, followed by a 3 min ramp to 1 min of primer elongation at 72°C. The final primer elongation segment was extended to 9 min. The reaction products were analyzed on agarose gels containing 1% FMC Seakem agarose and 1% FMC NuSieve agarose. Gels were visualized with ethidium bromide and progeny were scored by presence or absence of specific parental bands. Primer sequences and related informati on for primers used for mapping are shown in Table 1.

Linkage Analysis
Linkage analysis was performed using the computer programs LINKAGE-1 (Suiter et al. 1983) and MapMaker (Lander et al. 1987). Initial linkage analysis was performed with the version of MapMaker for the Macintosh computer. Linkage groups were identified with the "group" command and three point analysis was performed with a LOD score of 3.0. Haldane recombination distances were used to generate the map (Figure 1). LINKAGE-1 was used to confirm linkages found with MapMaker by pairwise comparison, and also used to perform goodness-of-fit tests to analyze segregation ratios.

Results and Discussion

Polymorphism Levels
One of the goals in this work was to compare the organization of the genome of chickpea with the genomes of pea and lentil, which are more extensively described. We therefore chose to focus on probes that have been mapped in those other closely related legume crops. We were limited primarily to the probes developed by Havey and Muehlbauer (1989), which have been used for both lentil (Havey and Muehlbauer 1989; Weeden et al. 1992) and pea (Weeden and Wolko 1990). Twenty-six of these probes have been useful in lentil, and an additional 11 have been mapped in pea. This set of probes consists of both cDNA and random genomic sequences. Because levels of RAPD and RFLP polymorphism generally parallel levels of allozyme polymorphism, we chose to perform DNA polymorphism analysis of interspecific crosses of chickpea because of the reported paucity of isozyme polymorphism in *C. arietinum* (Oram et al. 1987; Tuwafe et al. 1988). Our own results soon con-

Table 1. Sequences of the primers used to map the *Cicer* genome

Primer	Sequence	Locus	Parental source	Approx. size (bp)
CS4	GACTTCCTGT	C	*C. arietinum*	1,900
CS5	CCGGCTCTTG	A	*C. reticulatum*	600
		C	*C. reticulatum*	1,100
		D	*C. arietinum*	2,000
CS15	AACACATGCC	A	*C. reticulatum*	500
		B	*C. arietinum*	550
CS27	AGTGGTCGCG	A	*C. arietinum*	700
		C	*C. arietinum*	1,050
CS30	GCGTAGAGAC	B	*C. arietinum*	800
		D	*C. arietinum*	1,900
CS31	CTCGACACTG	C	*C. arietinum*	900
		D	*C. arietinum*	1,000
CS33	CAGTATTCGC	A	*C. arietinum*	300
		B	*C. reticulatum*	700
CS34	GATAGCCGAC	A	*C. arietinum*	500
		B	*C. arietinum*	1,000
		C	*C. reticulatum*	1,100
		D	*C. reticulatum*	1,800
		F	*C. reticulatum*	800
CS39	TCGGCCTGCT	A	*C. arietinum*	600
		C	*C. reticulatum*	1,750
CS44	ATTCGGCCGC	A	*C. arietinum*	350
		B	*C. reticulatum*	750
		C	*C. arietinum*	900
		D	*C. arietinum*	1,200
CS45	CACGTCGGAG	B	*C. reticulatum*	1,100
CS46	GGGATCTAGC	C	*C. arietinum*	500
CS47	TTGCCGTGTT	A	*C. reticulatum*	300
		B	*C. arietinum*	350
CS48	CTCTGCTTAG	A	*C. reticulatum*	1,000
		B	*C. arietinum*	1,800
CS53	GCCTCATACC	A	*C. arietinum*	900
		B	*C. arietinum*	1,200
CS54	AAGCGATGTT	A	*C. arietinum*	500
		C	*C. reticulatum*	1,000
		D	*C. reticulatum*	1,300
CS56	TGGTGGGTCC	C	*C. arietinum*	1,700
CS61	GAAAGGACGC	A	*C. reticulatum*	400
		B	*C. reticulatum*	1,600
		C	*C. reticulatum*	2,000
CS62	GATCCGCGTG	A	*C. reticulatum*	300
CS64	AACTGGCGAC	B	*C. arietinum*	500
CS65	TTGCTAGGGG	A	*C. reticulatum*	400
CS66	GCTCACCCTA	A	*C. reticulatum*	200
		B	*C. arietinum*	900

The parental source of the signal and the approximate molecular weight of the band(s) polymorphic in the crosses are listed after each primer locus designation.

firmed this observation, as an examination of 26 RFLP probes on six enzyme cuts revealed total monomorphism for single copy sequences among all of the *C. arietinum* parents. The only RFLP polymorphisms we found within the species were for a minor band that appeared with an rDNA sequence probe, and one or two polymorphisms of minor bands of moderately repetitive sequences that we did not feel we could score reliably. Even when surveying the interspecific parents we found low levels of polymorphism. We had good success in hybridizing nearly all of the cDNA probes to chickpea DNA, but we were only able to detect polymorphism between the interspecific parents with 10 of the probes. In a few cases we extended our enzyme cuts to 12 different enzymes, but this yielded no additional polymorphism. The lentil random genomic sequences presented an additional problem, in that very few of them hybridized effectively to the chickpea DNA. About half of these probes were from a *Pst*I library, while the other half were from an *Eco*RI library. The *Pst*I clones were somewhat better at hybridizing to chickpea DNA, but only one of the clones was useful as a mapping probe. In nearly all cases, the signal from these probes washed off with the background, even under the low stringency washing conditions described above. This lack of homology suggests considerable divergence between lentil and chickpea noncoding DNA.

In examining the three interspecific *Cicer* populations with the RAPD technique, we found levels of polymorphism similar to isozyme and RFLP results. RAPD polymorphism was present between *C. arietinum* parents in very few cases. We regarded the apparent lack of polymorphism in the chickpea genome to be an advantage rather than a problem, however, for the following reason. Since RAPD technology is still relatively new, there is considerable concern about the risks and the limitations of this technique as a mapping tool (numerous personal communications). One viewpoint commonly expressed suggests that one must be especially cautious in attempting to compare results from one cross with results from another, because RAPD bands of the same mobility may not represent the same sequence. While this viewpoint is certainly expedient in many cases, we believe that the stability of the chickpea genome affords some relief from this potential problem. We have been able to score RAPD polymorphisms from all three of our mapping populations and independently perform linkage analysis of each population. When comparing the results of the analysis, very few anomalies occurred, which, besides increasing our confidence in the linkages we found, suggests that RAPD data are generally transferable across chickpea crosses. This high degree of consistency is an especially favorable circumstance in the context of our eventual desire to utilize RAPD polymorphisms as markers in applied chickpea breeding programs.

For many of the loci mapped, we found different polymorphisms among the three crosses with both RAPD and RFLP analysis. The *C. arietinum* parents were uniform for each of these loci, while the differences were always derived from the *C. reticulatum* parent. This was true despite the fact that the same P.I. accession (P.I. 489777) was used in each cross. When we looked at a random selection of 12 seed of this accession with several of the markers that mapped differently between the crosses, we found a high level of genetic polymorphism within this accession of *C. reticulatum* (Simon CJ and Muehlbauer FJ, unpublished result). It is therefore apparent that different genotypes of the wild parent were used for the three crosses, resulting in different sets of loci segregating among the crosses. Colinearity of markers never differed significantly between the crosses, but map distances sometimes were different. Ellis et al. (1992) also encountered different map distances in their attempt to compile a consensus map of the pea genome. Complex alignment diagrams presented by those authors illustrate the caution they give in performing such compilations.

The paucity of polymorphism in any single cross in our study, however, has frustrated our attempt to establish a suffi-

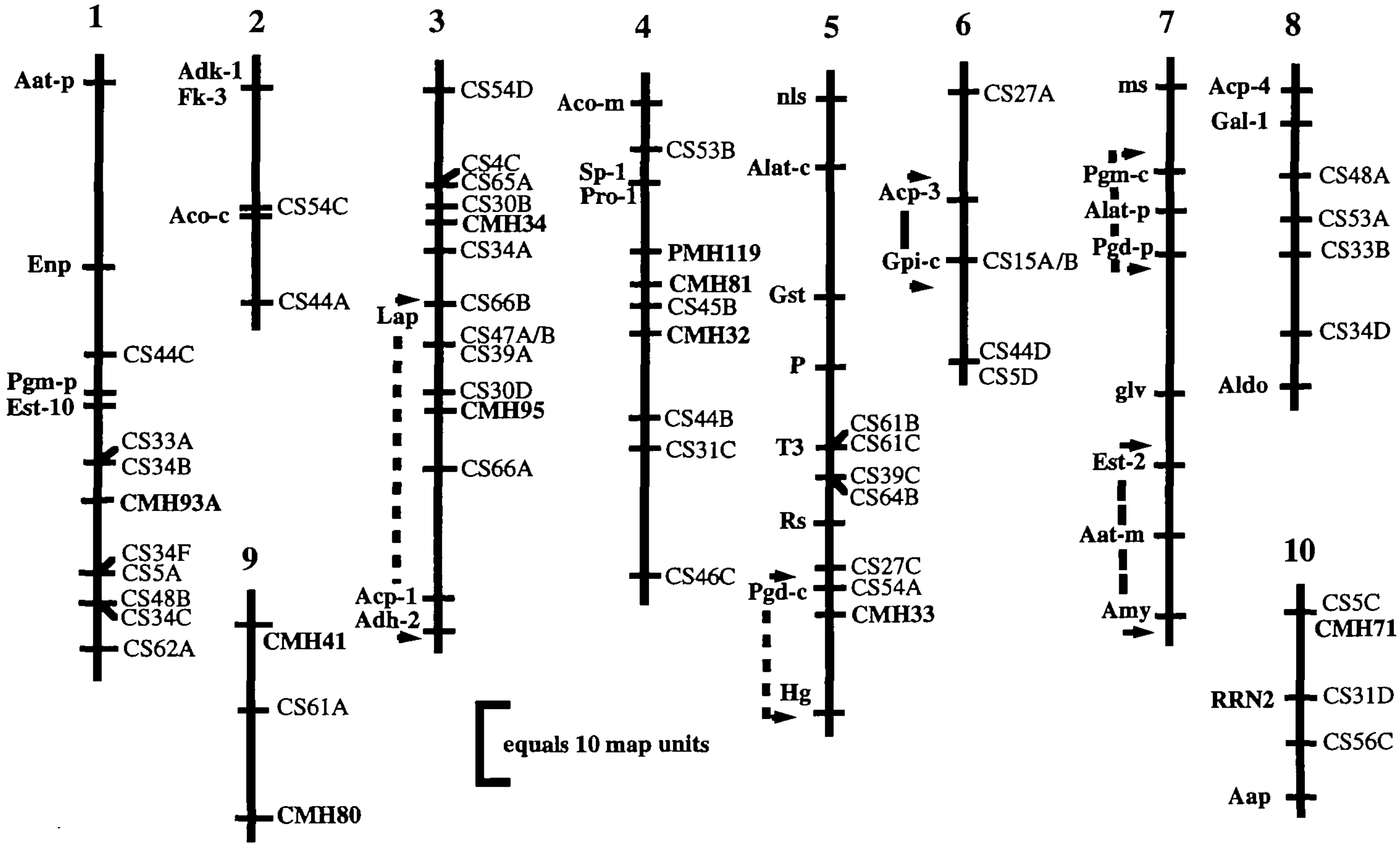

Figure 1. Linkage map for *Cicer*. Integrated map generated from three interspecific crosses of *C. arietinum* × *C. reticulatum*. Gene symbols shown to the left of the linkage segments are isozyme and morphological markers. To the right of the segments are RFLP (those with "MH" in the prefix) and RAPD (those with "CS" in the prefix) markers. Markers that have been used in pea and have similar arrangements in *Cicer* are highlighted by a dotted line next to the conserved region.

ciently dense map from one cross. This required us to merge data from the three crosses. The raw data used to establish our map are available on the World Wide Web for examination by interested persons. The USDA National Agriculture Library Agricultural Genome Information Server (AGIS) contains a module called "CoolGenes," which is a database presentation of genome maps and related information for cool season food legumes. Map loci are hypertext, which, when selected, describe the locus and report its mapping data, such as segregation ratios, and goodness-of-fit analysis, probe/enzyme combinations, etc.

Intergenic Comparisons

A compiled map of chickpea including morphological, isozyme, RFLP, and RAPD markers is shown in Figure 1. Comparison of this map to similar maps for pea (Weeden et al. 1993) and lentil (Havey and Muehlbauer 1989; Muehlbauer et al. 1989; Weeden et al. 1992) reveal at least five genomic regions that resemble each other. Assignment of markers to specific chickpea chromosomes was patterned after the pea map, which is the most advanced map of these genera. Conserved groups in Figure 1 are indicated with a heavy dotted line. The conserved group indicated on linkage group 3 resembles the pea group reported by Weeden et al. (1993) that includes the isozyme loci *Lap-1, Acp-3,* and *Adh-1.* A subset of this group may also be shared with linkage group 6 of lentil, which associates *Lap-1* and *CMH-95* (Weeden et al. 1992). Linkage group 5 of the chickpea map shown in Figure 1 contains a small group described by Kazan et al. (1993) that may also be found in pea. Chickpea linkage group 6 contains a small segment, consisting of two isozyme loci, that is similar to a segment found in pea (Weeden et al. 1993). Linkage group 7 in chickpea shows the greatest similarity to groups found in the other two genera. At the top of the chickpea group is a segment that is also found in group 7 of pea (Weeden et al. 1993) and group 5 of lentil (Weeden et al. 1992). At the bottom of the chickpea linkage group is a region resembling that found in pea (Weeden et al. 1993).

Weeden et al. (1992) found eight regions that showed similarity between the lentil and pea maps. This higher degree of similarity is consistent with the taxonomic relationship between these genera, since pea and lentil are in the same tribe (Viceae), while chickpea, which had been in Viceae, has recently been reclassified as the monogeneric tribe, Cicereae. The fact that the basic chromosome number of chickpea is eight, while pea and lentil have seven chromosomes, is another indication of the degree of genetic difference between *Cicer* and the other two genera.

The degree of conservation of genomic arrangement among chickpea, pea, and lentil that we have verified so far offers hope that the comparison of these genomes will expedite the further elucidation of their genomic architectures. Hart and Langston (1977) showed that isozyme loci arrangements in wheat were also found in many relatives of wheat, while RFLP markers in potato resemble those of tomato quite closely (Bonierbale et al. 1988). While such high levels of conservation are not always the case, as with tomato and pepper (Tanksley et al. 1988), Weeden et al. (1992) have already shown that nearly 40% of the lentil map arrangements can be found in pea; while this number may not be as high in chickpea, our work has shown that there is considerable overlap of genomic arrangement. The practical implication of this is that we may be able to use conserved portions of the genome of pea, which is much more well defined than that of its relatives such as chickpea, as a model that we can use as a predictor for these other genomes. Considering the investments involved in developing detailed linkage maps, such clues may have great value.

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